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Diversity in peritoneal macrophage response of CAPD patients to 1,25-dihydroxyvitamin D_3

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Diversity in peritoneal macrophage response of CAPD patients to 1,25-dihydroxyvitamin D_3. A major complication of continuous ambulatory peritoneal dialysis (CAPD) is peritonitis. Increasing the activity of the peritoneal macrophages, the predominant cell type found in the peritoneal cavity, may be a promising treatment for this infection. The effect of 1,25-dihydroxy-vitamin D_3 [1,25$(OH)_2D_3$] on the activity of peritoneal macrophages from CAPD patients and nonuremic controls was studied. 1,25$(OH)_2D_3$ had a biphasic effect on superoxide generation in the concentration range of 2.5 10^{-9} M to 5 $\times$ 10^{-6} M with a peak at 2 $\times$ 10^{-8} M. The addition of 2 $\times$ 10^{-8} M 1,25$(OH)_2D_3$ to nonuremic control macrophages for 24 hours caused a significant twofold increase in superoxide generation in response to phorbol myristate acetate (PMA), from 2.21 + 0.2 to 4.1 + 0.2 nmol/10^6 mac ($P < 0.001$), and enhanced the bactericidal activity from 60 + 7% to 85 + 9% ($P < 0.005$). CAPD patients were divided into two groups: Group A, patients with high peritonitis incidence (HPI); group B, patients with low peritonitis incidence (LPI). Macrophages from HPI patients show a lower bactericidal activity (37 ± 5%) and were not affected by 1,25$(OH)_2D_3$ after 24 hours of treatment. The increase in macrophage activity was seen only after three days of incubation with the hormone. Macrophages from this group generated a high amount of prostaglandin E_2 (PGE_2) during the first 24 hours in culture (7.8 ± 0.52 ng/ml as compared with 0.35 ± 0.03 ng/ml in the controls). Addition of 1,25$(OH)_2D_3$ together with indomethacin (10^{-6} M) enhanced the effect of 1,25$(OH)_2D_3$ on the macrophages from these patients even after 24 hours of incubation. However, macrophages from LPI patients behaved similarly to macrophages from control subjects. Incubation of 1,25$(OH)_2D_3$ with the macrophages for 24 hours significantly increased superoxide generation from 2.47 ± 0.2 to 3.86 ± 0.35 nmol/10^6 mac ($P < 0.001$), and killing activity from 62.5 ± 4 to 83 ± 6% ($P < 0.001$). The concentration of PGE_2 (0.37 ± 0.045 ng/ml) released after 24 hours in culture was similar to that of the control. The addition of PGE_2 with 1,25$(OH)_2D_3$ to macrophages from LPI patients prevented the increase in macrophage activity caused by 1,25$(OH)_2D_3$. These results indicate that PGE_2 has a role in modulating the effect of 1,25$(OH)_2D_3$ on the activity of peritoneal macrophages from CAPD patients.

Renal replacement therapy for end-stage renal disease has developed rapidly during the last decade, with an increasing number of patients utilizing continuous ambulatory peritoneal dialysis (CAPD) [1]. However, even today bacterial peritonitis remains a major complication in CAPD patients [2]. The defective bactericidal capacity of peritoneal macrophages could be responsible for the reduction in defense against microorganisms and for the high incidence of peritonitis [3]. Mononuclear phagocytes constitute the predominant cell type found in the peritoneal dialysate from non-infected patients undergoing acute or chronic peritoneal dialysis [4, 5]. Between 70% and 80% of the cells obtained from dialysis effluents have the characteristics of peritoneal macrophages [6]. Thus, peritoneal mononuclear phagocytes are the first line of cellular defense against microorganism invasion in CAPD patients. An increase in macrophage activity could be important in the prevention and therapy of peritonitis.

The hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] primarily affects cells in the intestine, bone and kidney, where it binds to specific cytosolic receptors [7, 8]. Recently, significant numbers of receptors for 1,25$(OH)_2D_3$ have been found in both normal and neoplastic cells, particularly monocytes and activated T-lymphocytes [9, 10]. There is also evidence for the role of 1,25$(OH)_2D_3$ in modulating functions of monocytes and macrophages in inflammation and immune cell reactions [11–13]. The aim of the present work was to study the effect of 1,25$(OH)_2D_3$ on the activity of peritoneal macrophages from CAPD patients.

Methods

Drugs

1,25$(OH)_2D_3$ used in this study was provided by Hoffmann-La Roche (Basel, Switzerland). The compound was reconstituted in ethanol and stored in concentrated solutions at −20°C. The vitamin D metabolite was freshly diluted in the appropriate medium before each experiment. The ethanol concentration under the test conditions and in the controls did not exceed 0.1%.

PGE_2 and indomethacin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The indomethacin was prepared by first dissolving it in a small volume of 95% ethyl alcohol and then bringing it to 10^{-3} M in Hanks Buffer Saline Solution (HBSS). It was then titrated to pH 7 with $NaHCO_3$.

Patients

Twenty patients who had been receiving CAPD for 16.3 ± 1.4 months (range 6 to 30 months) were selected. Patients with

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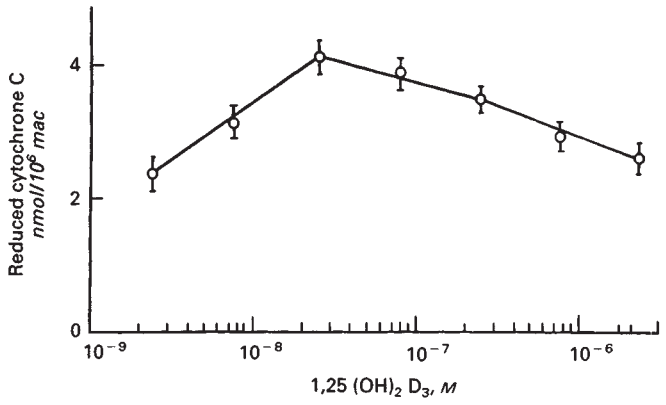

Fig. 1. *Dose response effect of 1,25(OH)$_2$D$_3$ on the reduction of cytochrome C stimulated by PMA in human peritoneal macrophages from control donors.* The results are mean ± SE from three donors. The reduction of cytochrome C stimulated by PMA without addition of 1,25(OH)$_2$D$_3$ was 2.3 ± 0.35.

diabetic nephropathy were not included in the study. The patients had been free of peritonitis for at least three months at the time of the study. CAPD was performed according to the procedure described by Oreopoulos et al [14]. The dialysate solution (Dianeal, Travenol, Israel) contained following: 132 sodium, 3.5 calcium, 1.5 magnesium, 102 chloride and 35 lactate (mEq/liter) and glucose concentration of 1.5%. All patients were dialyzed through a permanent double-cuffed Teuckhoff catheter. None of the patients undergone parathyroidectomy or renal transplant. The patients were divided into two groups: A — patients with a high incidence of peritonitis (HPI), in whom peritonitis occurred at a rate of more than one episode every six patient-months; B — patients with a low incidence of peritonitis (LPI), in whom peritonitis occurred at an overall rate of less than one episode every 12 patient-months. All patients received oral supplementation of calcitriol at doses ranging from 0.25 to 2.5 μg per day. Both groups of patients were matched for age, sex and etiology for chronic renal failure.

Peritoneal macrophages from 10 control subjects with normal renal function were obtained from peritoneal lavage during elective laparotomy. The CAPD patients and controls were age and sex matched.

Cell preparation

Peritoneal macrophages were obtained from CAPD patients as described by Goldstein et al [5]. The complete dialysate effluent volume from an overnight exchange was centrifuged at 150 × *g* for 20 minutes). Cells were washed twice in RPMI-1640 medium (Bet Haemek, Israel) and resuspended in RPMI medium which contained 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin and 12.5 U/ml nystatin (Biological Industries, Bet Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Final cell suspensions had a mean composition of 85% macrophages, 10% lymphocytes, 3% neutrophils, and 2% eosinophils, as identified by morphology on Wright-Giemsa staining. Samples containing more than 5% neutrophils were not included in this study.

Macrophage monolayers were prepared by placing 1 ml (1 × 10^6 cells/ml) onto plastic tissue culture dishes (35 × 10 mm, Falcon Plastics, Oxnard, California, USA), and allowing for macrophage attachment for 90 minutes at 37°C in a humidified incubator (5% CO_2 in air). The monolayers consisted of 95% monocytes as determined by monocyte-specific monoclonal antibodies against mac 1 [15]. More than 95% macrophages were viable as determined by trypan blue exclusion.

Superoxide anion production

The superoxide dismutase-inhibitable reduction of cytochrome C was assayed as described by Johnston, Godzik and Cohen [16]. Suspensions of 10^6 macrophages in HBSS were placed onto plastic tissue culture dishes (35 × 10 mm, Nunc) containing 80 μM ferricytochrome C (Sigma). The reaction was initiated with the addition of 10^{-7} M phorbol myristate acetate (PMA). Fifty μg/ml superoxide dismutase (SOD) was added to the reference dishes. The cells were incubated in a humidified incubator at 5% CO_2 in air at 37°C. The optical density of the supernates was measured spectrophotometrically and the O_2^- released was determined as SOD-inhibitable reduction of cytochrome C using the extinction coefficient $E_{550} = 2.1 \cdot 10^4$ M^{-1} cm^{-1} and expressed for 15 minutes.

Assay of bactericidal activity of peritoneal macrophages

Bactericidal activity of peritoneal macrophages for *Staphylococcus epidermidis* (*S. epidermidis*) was tested using a standard method [17]. *S. epidermidis* were grown for 18 hours at 37°C in a broth containing 1% peptone, 0.5% yeast extract (Difco) and 0.5% NaCl. The bacteria were washed three times in phosphate buffer saline (PBS), pH 7.4 and adjusted to the final concentration by means of a spectrophotometric method, the results of which were confirmed by plate colony counts. Bacterial suspensions (0.1 ml, containing 10^7 microorganisms on the average, as defined spectrophotometrically) were preopsonized with 0.9 ml 10% pooled normal serum in HBSS for 60 minutes at 37°C. The suspensions were centrifuged and the bacterial pellets were resuspended in 1 ml of HBSS (preopsonized bacteria). One hundred microliters of preopsonized bacteria was mixed with an equal volume of peritoneal macrophage suspensions (10^6 cells/ml of HBSS) in polypropylene counting tubes. The ratio of bacteria to peritoneal macrophages was 10:1. Immediately after the mixtures were made (0 time), 1, 2 and 3 hours after incubation in a shaking incubator at 37°C, serial tenfold dilutions in sterile water were performed. Triplicate 20 μl samples from appropriate dilutions were plated on nutrient agar, and bacterial colonies were counted after a 24-hour incubation period at 37°C. In all experiments, control tests were performed by incubating bacteria without macrophages. The results were expressed as percentage of colony forming units (CFU) killed (percentage decrease in the number of CFU after the incubation periods). Unless sited, the percent of killing was expressed after three hours of incubation.

PGE_2 assay

For PGE_2 assay, monolayers of 10^6 peritoneal macrophages in 16 nm culture dishes were incubated in 1 ml culture medium for 24 hours.

PGE_2 release in culture supernatants of the peritoneal macrophages was determined by radioimmunoassay (New England Nuclear, Boston, Massachusetts, USA).

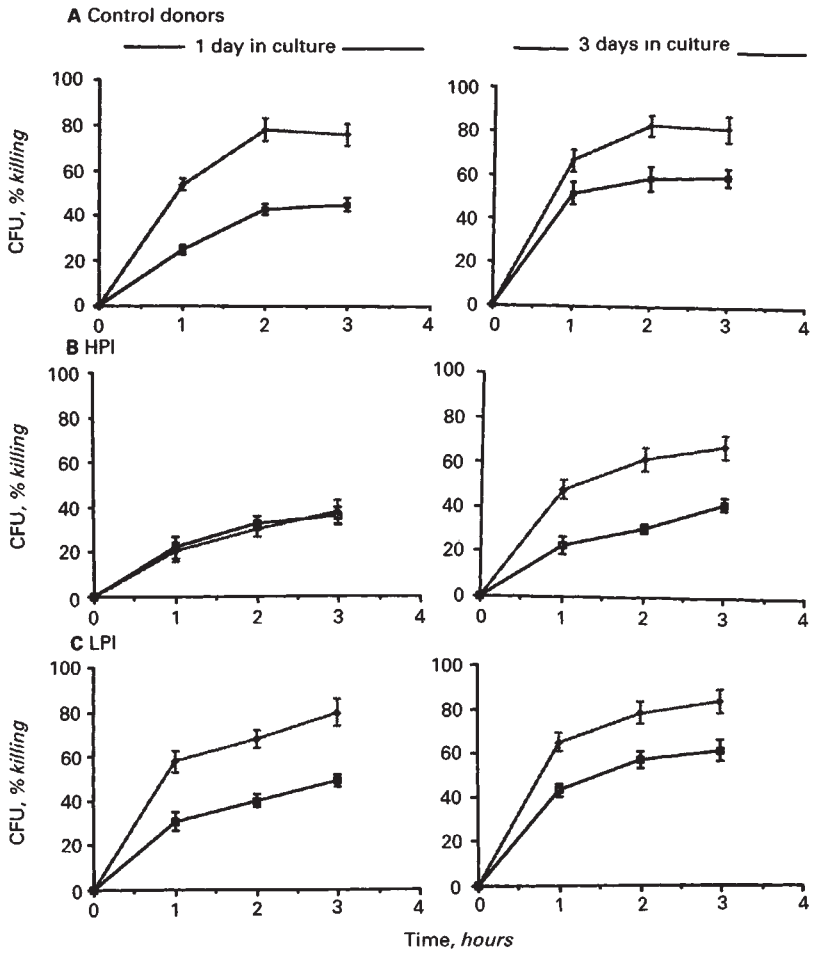

Fig. 2. *Effect of $1,25(OH)_2D_3$ on the killing of S. epidermidis by peritoneal macrophages.* **A.** Control donors. **B.** HPI-CAPD patients with a high incidence of peritinitis. **C.** LPI-CAPD patients with a low incidence of peritinitis. The concentration of $1,25(OH)_2D_3$ given was 2×10^{-8} M. Symbols are: (⊡) untreated macrophages; (◆) $1,25(OH)_2D_3$-treated macrophages. The results are mean ± SEM from 5 donors in each group.

Statistical analysis

The results are given as mean + SEM. Significance was calculated using the Student's paired *t*-test. Significance between the groups was calculated using analysis of variance.

Results

Figure 1 describes the dose response effect of $1,25(OH)_2D_3$ on superoxide generation in response to 10^{-7} M PMA as measured by cytochrome C reduction, in human peritoneal macrophages from healthy donors. Incubation of the cells with $1,25(OH)_2D_3$ for 24 hours caused a biphasic effect on cytochrome C reduction within the range of 2.5×10^{-9} M to 5×10^{-6} M. The optimum effect was achieved at 2×10^{-8} M $1,25(OH)_2D_3$, which caused a twofold increase in the activity, from 2.21 ± 0.2 in the control to 4.1 ± 0.2 nmol/10^6 mac ($P < 0.001$). Incubation of peritoneal macrophages from normal donors ($N = 10$) with $1,25(OH)_2D_3$ (2×10^{-8} M) for 24 hours significantly increased their killing activity from 60 ± 10% to 85 ± 9% ($P < 0.005$). The same concentration of $1,25(OH)_2D_3$ (2×10^{-8} M) was found to cause the maximal effect in macrophages from CAPD patients as well. Thus, in all further experiments this concentration of $1,25(OH)_2D_3$ was used.

Figure 2 describes the effect of $1,25(OH)_2D_3$ (2×10^{-8} M) on the killing activity of peritoneal macrophages from normal donors and from CAPD patients. Incubation of the macrophages from normal donors with $1,25(OH)_2D_3$ for one or three days caused an increase in the killing activity. One day of treatment with $1,25(OH)_2D_3$ increased the killing from 45 ± 4% to 76 ± 5% and three days of treatment increased it from 60 ± 4% to 81 ± 6% ($P < 0.005$). Treatment of the peritoneal macrophages from HPI- CAPD patients with $1,25(OH)_2D_3$ for 24 hours did not affect the killing activity. Only after three days of treatment of the macrophages with $1,25(OH)_2D_3$ did the killing activity increase from 42 ± 3% in the untreated cells to 68 ± 5% ($P < 0.001$). In LPI-CAPD patients, the peritoneal macrophages behaved in a similar manner to the peritoneal macrophages from normal donors. As shown in Figure 2, the addition of $1,25(OH)_2D_3$ to the macrophages for one or three days caused a significant increase in their killing activity ($P < 0.001$).

Figure 3 summarizes the release of PGE_2 from 10^6 resting macrophages cultured for 24 hours. The PGE_2 concentration released from the peritoneal macrophages of normal donors and from LPI-CAPD patients belonging to group B was 0.35 ± 0.03 and 0.37 ± 0.045 ng/ml, respectively. The concentration of PGE_2 released from peritoneal macrophages from HPI-CAPD patients was much higher, reaching a level of 7.8 ± 0.52 ng/ml after 24 hours in culture. The amount of PGE_2 secreted from the macrophages of the three groups decreased gradually during growth in culture. The most pronounced reduction was in the

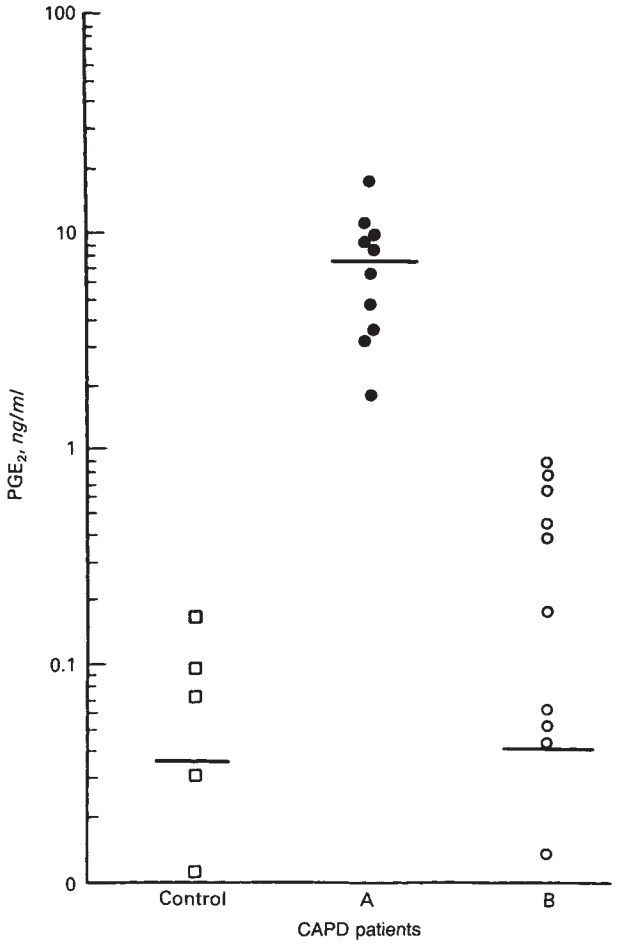

Fig. 3. *Concentration of prostaglandin E_2 (PGE_2) released from peritoneal macrophages during 24 hours of culture.* C—control donors, A—HPI CAPD patients, B—LPI CAPD patients. Vertical lines indicate the mean values.

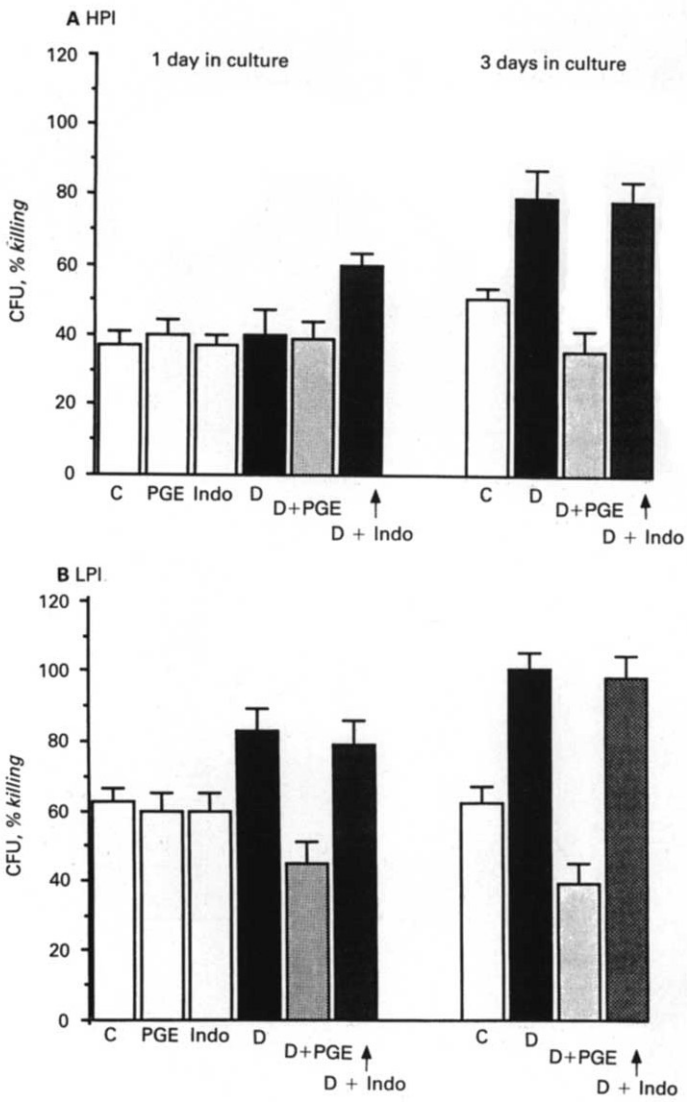

Fig. 4. *Effect of $1,25(OH)_2D_3$, prostaglandin E_2 and indomethacin on the killing of S. epidermidis by peritoneal macrophages from CAPD patients.* Abbreviations are: HPI, CAPD patients with a high incidence of peritonitis; LPI, CAPD patients with a low incidence of peritonitis; D, $1,25(OH)_2D_3$; PGE, prostaglandin E_2; Indo, indomethacin. The concentration of $1,25(OH)_2D_3$ is 2×10^{-8} M, PGE_2 = 10 ng/ml and indomethacin 10^{-6} M. The results are the mean ± SE of 10 patients in each group.

macrophages from group HPI-CAPD patients, from 7.8 ± 0.52 ng/ml after one day in culture, to 0.5 ng/ml after three days of incubation.

To study the role of PGE_2 in controlling the effect of $1,25(OH)_2D_3$ on macrophage activity, the effect of the addition of PGE_2 or indomethacin to the peritoneal macrophages during the treatment with $1,25(OH)_2D_3$ was studied. Figure 4 summarizes the killing activity of peritoneal macrophages from CAPD patients. The killing by untreated macrophages from HPI group was 37.2 ± 5%. Addition of $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ together with PGE_2 (10 ng/ml) for 24 hours to the macrophages from this group did not affect the rate of killing. However, the addition of indomethacin (10^{-6} M) together with $1,25(OH)_2D_3$ for 24 hours caused a significant increase in the killing activity to 60 ± 3% ($P < 0.001$). In LPI group, the killing activity was similar to normal controls (62.5 ± 4% and 60 ± 7%, respectively). Addition of $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ with indomethacin to the macrophages for 24 hours increased the killing to 83.6 ± 6 and 79.2 ± 7%, respectively, while the addition of PGE_2 to $1,25(OH)_2D_3$ decreased the killing to 45 ± 5.5% ($P < 0.005$). The killing activity and the effect of $1,25(OH)_2D_3$ was similar for both groups after three days of treatment with the vitamin with or without the presence of indomethacin or PGE_2.

The effect of PGE_2 and indomethacin on superoxide generation by the peritoneal macrophages during the treatment with $1,25(OH)_2D_3$ (Fig. 5) showed a similar pattern to that of the killing experiments. In HPI-CAPD patients, the addition of $1,25(OH)_2D_3$ to the culture for 24 hours did not affect the level of superoxide generation in macrophages stimulated by PMA. Indomethacin added together with $1,25(OH)_2D_3$ caused a slight but significant augmentation of superoxide generation from 2.2 ± 0.1 to 2.47 ± 0.13 nmol/10^6 mac ($P < 0.001$). The addition of PGE_2 alone or together with $1,25(OH)_2D_3$ caused a slight decrease in the activity, to 1.59 ± 0.26 and 1.77 ± 0.13 nmol/10^6 mac, respectively. However, a significant effect of $1,25(OH)_2D_3$

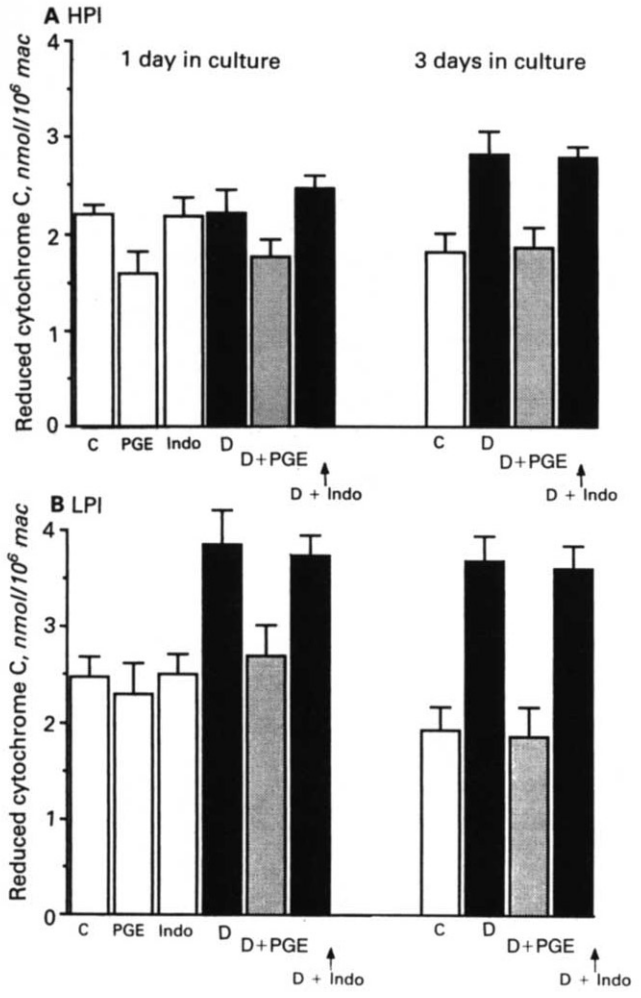

Fig. 5. *Effect of 1,25(OH)$_2$D$_3$, prostaglandin E$_2$ and indomethacin on superoxide generation in peritoneal macrophages stimulated by PMA.* Abbreviations are: HPI, CAPD patients with a high incidence of peritonitis; LPI, CAPD patients with a low incidence of peritonitis; D, 1,25(OH)$_2$D$_3$; PGE, prostaglandin E$_2$; Indo, indomethacin. The concentration of 1,25(OH)$_2$D$_3$ is 2×10^{-8} M, PGE$_2$ = 10 ng/ml, indomethacin = 10^{-6} M, and PMA = 10^{-7} M. The results are the mean ± SE of 10 patients in each group.

in increasing superoxide generation was shown after three days of incubation: from 1.82 ± 0.2 nmol/10^6 mac in the untreated macrophages to 2.83 ± 0.26 nmol/10^6 mac ($P < 0.001$). In LPI patients, the addition of 1,25(OH)$_2$D$_3$ to the macrophages for 24 hours caused an increase in superoxide generation from 2.47 ± 0.22 nmol/10^6 mac in the untreated cells to 3.86 ± 0.35 nmol/10^6 mac ($P < 0.001$). When PGE$_2$ was added together with 1,25(OH)$_2$D$_3$, it abolished the effect of 1,25(OH)$_2$D$_3$ and cytochrome C reduction levels were similar to the basal level of the untreated cells, while the addition of indomethacin did not change the effect of 1,25(OH)$_2$D$_3$. Similar results were obtained after three days of incubation.

Discussion

The present study demonstrates that 1,25(OH)$_2$D$_3$ (2×10^{-8} M) increases superoxide generation and killing activity of peritoneal macrophages from normal subjects. Incubation of the peritoneal macrophages with 1,25(OH)$_2$D$_3$ for three days restored their capacity to generate superoxide (Fig. 5), while untreated macrophages lost 25% of their activity after three days in culture. The mechanism by which 1,25(OH)$_2$D$_3$ preserves the superoxide generation and enhances phagocytic cell activity is not clear. A similar concentration of 1,25(OH)$_2$D$_3$ has been shown by other investigators to cause maximal stimulation of different monocyte activities [18]. Several studies have reported, in accordance with our results, that peripheral blood monocytes treated with 1,25(OH)$_2$D$_3$ for three days demonstrated a significant increase in H_2O_2 secretion relative to control cells [19–21]. Goldman [22] has shown that 1,25(OH)$_2$D$_3$ interacts specifically with a macrophage-like murine tumor cell line P388D1 and increases their phagocytic capability. They have suggested that 1,25(OH)$_2$D$_3$ may modulate the differentiation of early macrophage precursors, thus producing a population which expresses a more mature phenotype than that expressed in control culture. This is supported by the work of Polla et al [23] who demonstrated that 1,25(OH)$_2$D$_3$ modulates the adherence of monocytes accompanied by relative preservation of total protein synthesis. Thus the increase in the superoxide generation activity by 1,25(OH)$_2$D$_3$, shown in the present study may be mediated by preservation of protein synthesis which is necessary for superoxide generation and killing activity.

The CAPD patients could be divided into two groups according to their peritoneal macrophage activity. The response to 1,25(OH)$_2$D$_3$ was well correlated (r = 95%) with the low amounts of PGE$_2$ released by the peritoneal macrophages. Thus the low activity and the lack of responsiveness of the macrophages to 1,25(OH)$_2$D$_3$ in HPI group could be attributed to the high release of PGE$_2$ which is known as a down-regulatory molecule [24]. A similar phenomenon was reported by other investigators [25, 26], showing that peritoneal macrophages from CAPD patients with a high incidence of peritonitis are characterized by a decreased ability to generate oxygen metabolites and to kill bacteria, and by a lack of IgG Fc-receptor expression. Similar to the effect of 1,25(OH)$_2$D$_3$ demonstrated in our work, it was shown [25] that the defects could be cured after treatment with interferon-γ. In addition, they have shown that macrophages from HPI-CAPD patients which were unable to produce normal amounts of interleukin-1, released large amounts of PGE$_2$, decreased blastogenic response and reduced killing activity.

This study shows that the amount of PGE$_2$ released from the macrophages of HPI-CAPD patients diminished during growth in culture and thus, after three days, the effect of 1,25(OH)$_2$D$_3$ in enhancing the activity could be detected. The role of PGE$_2$ in modulating the effect of 1,25(OH)$_2$D$_3$ on macrophage activity was further supported by in vitro studies. The exogenous addition of PGE$_2$ prevented the increase in the activity caused by 1,25(OH)$_2$D$_3$ in CAPD patients with LPI, while the addition of indomethacin enabled 1,25(OH)$_2$D$_3$ to exert its enhancing effect even after one day of treatment in HPI-CAPD patients. The inhibitory effect of PGE$_2$ on macrophage activity has also been reported by others [27]. Non-stimulated cells in suspension demonstrated a dramatic elevation in PGE$_2$ production as compared to macrophage monolayers. This elevation in PGE$_2$ production was concomitant with the inability of macrophages in suspension to produce O_2^- or to undergo transmembrane

potential changes in response to PMA. Only after the macrophages became adherent did the elevated basal level of PGE_2 decrease, enabling the cells to respond to the stimulus. In addition, it was suggested [28] that the mechanism by which erythromycin mediates the increase in leukocyte migration is by inhibition of PGE_2 release from the cells.

The present study demonstrates that $1,25(OH)_2D_3$ increase both: superoxide generation and killing activity in peritoneal macrophage. In addition, the presence of PGE_2 or indomethacin together with $1,25(OH)_2D_3$ affect similarly both activities. These results may indicate the role of the respiratory burst in modulating killing activity. However, $1,25(OH)_2D_3$ is known to cause an enhancement of other functions, such as lysosomal activity and maturation of Fc receptors [18], which have a crucial role in increasing killing activity. Thus its effect in increasing the killing can be due to the increase of all these different activities. The fact that $1,25(OH)_2D_3$ affects several disparate functions suggests that these effects may be mediated by a general system of signal transduction.

The mechanism by which $1,25(OH)_2D_3$ enhances peritoneal macrophage activity as well as the role of PGE_2 in modulating its effect has yet to be studied. However, $1,25(OH)_2D_3$ treatment which results in an in vitro enhancement of macrophage functions, may be taken into consideration as a possible mode of therapy in CAPD patients.

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